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Atty. Docket No.: 11111/1210 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Segal	Examiner:	DeCloux, A
Serial No.:	09/318,870	Group Art Unit:	1644
Filed:	May 26, 1999		
Entitled:	Cytokine Coated Cells and Methods of Modulating an Immune Response to an Antigen	Conf. No.:	2018

CERTIFICATE OF FACSIMILE TRANSMISSION UNDER 37 C.F.R. § 1.6d

I hereby certify that this correspondence and any paper referred to as being enclosed are being facsimile transmitted on the date indicated above to the United States Patent and Trademark Office Fax No. (703) 305-3014

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Commissioner for Patents and Trademarks
Washington, D.C. 20231

RULE 132 DECLARATION OF DR. ANDREW SEGAL

I, Andrew Segal, hereby declare that:

1. I am the sole inventor of the above referenced patent application. I received a B.A. in Biochemical Sciences from Harvard University in 1985, and am M.D. from Boston University School of Medicine in 1990. I conducted postdoctoral research from 1994 to 1996 at the Whitehead Institute of Biomedical Research. I am currently the President and CEO of Genitrix, LLC.

2. I have read the Office Action mailed in the above-referenced patent application on October 20, 2002, and understand that questions have been raised by the Examiner as to whether the amounts of cytokine present in the vaccine of the present invention taught in the specification are effective to vaccinate a mammal to a selected antigen.

3. The experiments described herein (results shown in Exhibit A) were performed under my direction. These experiments were performed *in vivo* in mice. According to the invention, a fusion protein comprising the cytokine GM-CSF and a heterologous cell membrane binding

moiety is mixed with fibrosarcoma cells to produce a vaccine, prior to the administration of the vaccine to a mammal. The results demonstrate that the administration of a vaccine comprising a cytokine coated cell comprising GPI-linked GM-CSF mixed with fibrosarcoma cells, in an amount within the ranges taught in the specification, to a mouse inhibits tumor growth compared to the administration of a control vaccine which does not comprise a cytokine coated cell.

Exhibit A shows the results of an experiment following the protocols described hereinbelow, in which cytokine coated CMS-5 fibrosarcoma cells are used to vaccinate mice against subsequent CMS-5 tumor cell challenge. In these experiments, GPI-GM-CSF refers to a fusion protein comprising the cytokine GM-CSF fused to a heterologous membrane binding domain, glycosylphosphatidylinositol. The methods used to construct the GPI-GM-CSF have also been provided for completeness. The experiments described herein were performed as described in detail in the above-referenced patent application.

Construction of a prototype chimeric cytokine incorporating a glycosylphosphatidylinositol moiety, using GM-CSF as a framework

Cloning and Expression

The starting point for producing a GPI-GM-CSF expression vector was the pUC19 -GM-CSF-mammalian GPI signal sequence plasmid (pUC19 -GM-CSF-GPI) that had been constructed previously. This plasmid encodes murine GM-CSF (upstream) fused in-frame to the human Thy-1 GPI modification signal sequence (downstream). The Thy-1 sequence is flanked by a 5' NgoMIV restriction site and a 3' KpnI site. This plasmid was digested with NgoMIV and KpnI, and the larger resulting fragment isolated after electrophoresis through a 1% agarose gel.

The 280 bp GPI modification signal sequence from the yeast protein Gas1 was amplified by PCR from the yeast cosmid clone C9952 (ATCC). This PCR employed *pfu* polymerase and the primers:

Upstream Primer 5'GTAGCCGGCGCTAGCTCGGGGTCTTCTTCCAAGTCTA

Downstream

Primer 5'TACGGTACCCCTAGGCCACAATGAAATAAGATACCATACC3'

These primers add a 5' NgoMIV site and a 3' KpnI site to the Gas1 fragment.

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute
	Cycles	25	

The PCR product was purified after electrophoresis through a 1% agarose gel and digested with NgoM IV and KpnI. The Gas1 GPI signal sequence was then ligated into the pUC19-GM-CSF-GPI plasmid prepared above so that the Gas1 signal sequence was fused in-frame downstream of the GM-CSF sequence, replacing the Thy-1 sequence. This vector is termed pUC19-GMCSF-Gas1.1. The resultant plasmid was then transformed into AG-1 competent E. coli (Stratagene) and plasmid clones were isolated by alkaline lysis mini-prep. Plasmids were then screened for inserts by restriction digest. DNA from a positive clone was sequenced to confirm the identity of the *GAS1* coding region.

The yeast expression plasmid for GPI-GM-CSF was then generated utilizing the pITY-4 vector, which was kindly provided by Dr. K. Dane Wittrup (University of Illinois). This plasmid stably integrates into the yeast genome and allows high-level expression of heterologous genes. Features of pITY-4 include: a delta sequence (LTR of Ty element) that enables multiple integration events by homologous recombination; a *neo*/kanamycin resistance gene that provides for selection in E.coli and tunable selection in yeast; the Gal1 promoter for high-level inducible transcription; a unique EagI cloning site; a synthetic Pre-Pro sequence optimized for efficient secretion of expressed genes; the alpha factor termination sequence; and an origin of replication for propagation in E.coli. In this system, yeast are grown in dextrose-containing media for 3 days, then are switched to media containing galactose to induce transcription of genes inserted downstream of the Gal1 promoter.

The GMCSF-Gas1 insert described above was amplified by PCR from pUC19-GMCSF-Gas1.1 using *pfu* polymerase and the primers:

Upsteam 5'TACGGCCGGCACCCACCCGCTCACCC3'

Downstream 5'TACGGCCGCCACAATGAAAATAAGATACCAT3'

These primers add EagI sites at both ends for cloning into the pITY-4 plasmid.

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute

Extension	72^0	one minute
Cycles	25	

The PCR product was purified after electrophoresis through a 1% agarose gel and digested with EagI. The EagI-flanked GMCSF-Gas1 fragment was ligated into EagI-digested pITY-4 and used to transform E.coli AG1 cells. E. coli were then grown on kanamycin-containing LB plates (100 ug/ml). Plasmids from kanamycin resistant colonies were purified by mini-prep and mapped by restriction digests for presence and correct orientation of inserts. The identity of a positive clone was confirmed by sequencing. This plasmid is termed pITY-GMCSF-Gas1.1.

A 50 ml culture of the E.coli clone containing pITY-GMCSF-Gas1.1 was grown in LB with 100 ug/ml kanamycin and the plasmid purified using a Midi-Prep Kit from Qiagen. The *S. cerevesiae* strain BJ5464 (ATCC) was then transformed with pITY-GMCSF-Gas1.1 using a lithium acetate (LiAc) protocol. A 10 ml overnight culture of BJ5464 in YPD (Per liter: 20g Bactotryptone, 10g yeast extract, 20g dextrose) was used to inoculate a 100 ml flask. Yeast were grown for 3 hours at 30^0 and then harvested by centrifugation at 12,000 x g for 2 minutes at room temperature. Cells were washed with sterile water and centrifuged again. The cells were resuspended in 1.0 ml of 100mM LiAc, transferred to a 1.5 ml microfuge tube and centrifuged in an Eppendorf microfuge at top speed for 15 seconds. The cells were then resuspended in 0.5 ml of 100 mM LiAc and 50 uL samples were aliquoted to individual tubes. The cells were pelleted. 240 uL of PEG (50% w/v), 36 uL 1.0M LiAc, 5 uL (10 mg/ml) boiled carrier DNA (salmon sperm DNA, Sigma), and 2 ug plasmid in 75 uL water, were then added in that order. After the addition of plasmid, the tube was vortexed, incubated at 30^0 for 30 minutes and heat-shocked at 42^0 for 15 minutes. The cells were then pelleted, resuspended in sterile water and plated on YPD plates containing 1 mg/ml G418.

Individual colonies of G418-resistant yeast were picked and grown in one ml of YPD with 1 mg/ml G418 for 3 days. The cells were then pelleted by centrifugation in a microfuge and the YPD (dextrose-containing, galactose-free) media was replaced with YPG (20g bactotryptone, 10g yeast extract, 20g galactose per liter) with 1 mg/ml G418. Yeast were grown in YPG for 3 days to allow full induction of transcription from the Gal1 promoter. After induction, cells were pelleted, washed with TN (0.15M NaCl, 25mM Tris pH 7.4) and lysed in TN containing 20mM octyl glucopyranoside (OGP), 1 mM PMSF, and 1ug/ml each aprotinin, leupeptin and pepstatin.

Yeast were lysed by vortexing with acid-washed glass beads (425-600 microns, Sigma). Insoluble material was pelleted and the supernatant assayed using a murine GM-CSF ELISA (Endogen). A colony expressing high levels of GPI-GM-CSF was identified. Based on standard curve of soluble GMCSF, we estimate expression to be approximately 25 ug/L, a significant improvement over mammalian expression and sufficient for in vivo experiments. This yeast clone is designated SC-GM-GPI.

One of the advantages of stably integrating vectors for expression in yeast is that, after the initial cloning and colony isolation, antibiotic maintenance is no longer required. To confirm this, cells were grown with and without G418 and tested for GPI-GM-CSF expression. No decrease in expression levels in the absence of G418 has been observed for over 8 months.

Scale-Up, Purification, and Characterization of GPI- GM-CSF Expression

To produce GPI-GM-CSF on a scale suitable for *in vitro* and *in vivo* functional characterization, 500 ml of YPD was inoculated with SC-GM-GPI and grown for three days at 30^0 with shaking. Cells were pelleted by centrifugation at 12,000 x g for 2 minutes at room temperature and transferred to an equal volume of YPG for an additional three days of growth. Cells were then pelleted, washed with TN and lysed in 25 ml of TN containing 20mM OGP, 1 mM PMSF, and 1ug/ml each aprotinin, leupeptin and pepstatin. Cells were then lysed by vortexing with acid washed glass beads, 20g/ 500ml culture, (425-600 microns, Sigma). Insoluble material was pelleted at 8,000 x g for 10 minutes at room temperature and the soluble material was applied to an immunoaffinity column of anti-murine GMCSF monoclonal antibody (Endogen) linked to cyanogen bromide-activated Sepharose 4B (Sigma). Coupling of the monoclonal to the Sepharose was performed according to the manufacturer's instructions. Efficiency of coupling was monitored using OD_{280} and binding of murine GM-CSF to immobilized antibody was confirmed using commercially available, recombinant cytokine.

Soluble yeast-derived material was applied to the column and allowed to flow by gravity. The column was washed sequentially with: (a) 20 volumes of TN with 1% Triton X-100; (b) 5 volumes of 50mM Tris pH 8.0, 1mM OGP; (c) 20 volumes TN with 1mM OGP. Bound material was then eluted with 10 volumes of 0.15M NaCl, 25mM Tris pH 2.5 with 1mM OGP. Eluted material was neutralized with 1/200 volume of 1.5M Tris pH8.8. The purified material was concentrated using a Microsep 3K centrifugal device (Pall Gelman Laboratory). Yields of GPI-

GM-CSF were determined by ELISA (Endogen) to be 25 ug/L of culture. Final concentration was adjusted to 40 ug/ml by addition of 0.15M NaCl, 25mM Tris pH 7.4 with 1mM OGP.

Purified GPI-GM-CSF was analyzed by stained gel and western blot. Approximately 1ug of purified GPI-GM-CSF or recombinant soluble murine GM-CSF per lane were electrophoresed. Gels were then stained with silver nitrate using the Sigma silver staining kit according to the manufacturer's directions (Sigma). For western blots, gels were transferred to Protran BA83 (Schleicher and Schuell) using an Owl Scientific electric transblotter and blocked with TBS (Tris Buffered Saline) containing 0.05% Tween 20 and 2% nonfat dry milk overnight at room temperature. The blot was then incubated with primary antibody (rat monoclonal anti-murine GMCSF, Endogen) at 1:5000 dilution in blocking buffer for 2 hours at room temperature. The blot was washed with TBS-0.05% Tween 20, and incubated with a secondary antibody, alkaline phosphatase conjugated goat anti-rat IgG (Sigma) at 1:10,000 for 1 hour at room temperature. After washing, color was developed with NBT-BCIP (Sigma). A single dominant band migrating at approximately the same rate as a recombinant soluble GM-CSF standard is clearly present on both the gel and the blot (the molecular weight of the GPI moiety is only approximately 1500 [9] compared to approximately 14,000 for the protein moiety). Given the immunoreactivity with anti-GM-CSF and the ability of this material to bind to tumor cell membranes, these bands appear to represent GPI-GM-CSF. While some high molecular weight material, possibly representing aggregates, is visible in the blot, this material is not visible in the less sensitive silver stain, indicating that it is present in lower amount than the dominant band.

Determination of whether tumor cells mixed with GPI-GM-CSF can act as a preventive vaccine against a subsequent tumor challenge

These experiments included mice vaccinated with:

(a) Wild-type cells

(b) Cells incubated with soluble GM-CSF-Washed Following Incubation (total GM-CSF in dose: none)

(c) Cells incubated with soluble GM-CSF–Unwashed (total GM-CSF in dose: 1 microgram)

(d) Cells decorated with GPI-GM-CSF- Washed Following Incubation (total GPI-GM-CSF in dose: 0.74 nanograms by ELISA [mean of 2 experiments; 73 and 75 ng individually)

(e) Cells decorated with GPI-GM-CSF-Unwashed (total GM-CSF in dose: 1 microgram)

CMS-5 cells were grown to 70% confluence in DMEM, 10%FBS, Penicillin-streptomycin, harvested trypsinization, and washed 3 times with RPMI 1640. Viability was determined by trypan blue staining of an aliquot and the cells were then resuspended at a concentration of 4×10^6 cells/ml a 1 ul aliquots dispensed into siliconized microfuge tubes. The cells were incubated with 1 ug GPI-GM-CSF or 1 ug soluble recombinant murine GM-CSF per 10^6 cells for 3 hours at 37^0 C. "Washed" groups were then washed 3 times with PBS, 2 % FBS and resuspended at 4×10^6 cells/ml in RPMI 1640. An aliquot of the washed GPI-GM-CSF decorated cells was removed and the amount of cell-associated GM-CSF measured by ELISA as described above. We calculated that there were approximately 31,000 and 32,000 GPI-GM-CSF molecules/cell in the washed decorated groups in the two experiments, respectively.

The cells were irradiated at 3500 rads from a ^{137}Cs source. 8-10 week-old female Balb/c mice (which are syngeneic for CMS-5) were anesthetized by metofane inhalation and vaccinated subcutaneously in the left inguinal fold with 1×10^6 cells in 0.25 ml. Seven days later, wild-type CMS-5 cells at 70% confluence were harvested and washed 3 times in HBSS. Viability was determined by trypan blue staining of an aliquot and cells were adjusted to 4×10^6/ml in HBSS. The previously vaccinated mice were then injected subcutaneously behind the neck, under metofane anesthesia, with $2x\ 10^6$ live, wild-type CMS-5 cells in 0.5 ml HBSS.

Tumor development was assessed daily by palpation and visual inspection. "Onset" was defined as the first day on which a tumor mass was both palpable and visible. The observer was blinded to the vaccine received by each set of mice to ensure against bias. Mice were sacrificed by CO2 asphyxiation when tumors become unwieldy. Experiments were terminated 70 days after tumor challenge, as planned in advance. Since the 70 day duration was based on convention, and since many vaccinated mice survived to day 70 without a tumor (long after all other mice had developed tumors), we decided that using a *t*-test to analyze the results as continuous data and assigning long-term tumor-free mice an arbitrary 70-day value was not appropriate. We therefore analyzed the data as a binary value (i.e., tumor-free at day 70 vs. tumor onset before day 70) using the chi-square test (with Yates' correction for continuity since these are 2 x 2 comparisons *and* some values are less than 5).

Results are shown in Exhibit A. Data is pooled from three experiments for GPI-GM-CSF unwashed, soluble GM-CSF, and wild-type vaccine groups. Data for these groups includes that

from undepleted controls in a lymphocyte subset depletion experiment. Data for the GPI-GM-CSF vaccine group is pooled from two experiments, since this group was not included in the initial depletion experiment. The depletion experiment had 4 mice/group, and the other experiments had 5/group.

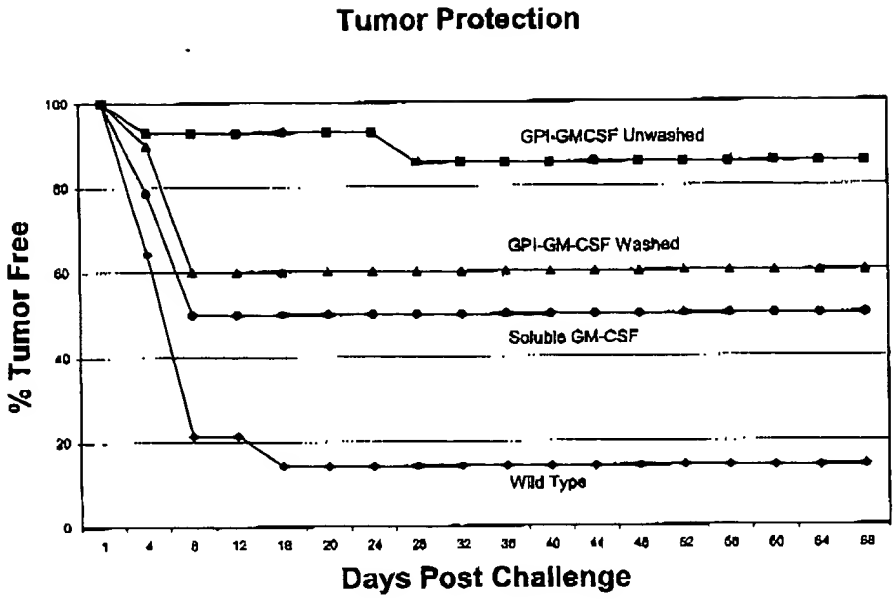
The effect of the washed GPI-GM-CSF vaccine on 70-day tumor free survival relative to the wild-type vaccine was slightly less than statistically significant at the 95% confidence level, but displayed a strong trend (p=0.057, chi-square= 3.621). Data from soluble GM-CSF washed vaccines (not shown) was consistently comparable to that from wild-type vaccines over three experiments. Over three experiments, the GPI-GM-CSF unwashed vaccine, which contained both cell-bound and free GPI-GM-CSF, consistently yielded 80-100% 70-day tumor-free survival. This was highly statistically significant relative to wild-type cells (p=0.001, chi-square=11.571). Of note, though, is the fact that less than one-thousandth of the amount of protein afforded more than 50% of this level of protection when delivered as membrane-bound cytokine.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

February 7, 2003
Date

Andrew Segal

EXHIBIT A



n = 14 for GPI-GM-CSF unwashed; 10 for GPI-GM-CSF washed; 14 for soluble GM-CSF (unwashed) and 14 for WT.